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ISOLATION AND CHARACTERIZATION OF
PLASMODIAL AND BABESIAL ANTIGENS

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in inducing immunity, and we have tentatively identified this soluble component as surface coat material. Thus our work on identification of protection stimulating antigens in Plasmodium benghei has been successful. The knowledge obtained from these studies must now be applied to development of a vaccine against malaria of man.

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The continuous flow sonic system developed in this laboratory (Prior and Kreier, 1972: 1972a) and various other methods for freeing malaria parasites from their host erythrocytes (Kreier, 1977) were evaluated from the standpoint of the morphological integrity of the freed parasites and the utility of the freed parasites as sources of antigen for serological tests. The antigens obtained from the parasites freed by the sonic system were evaluated by complement fixation, passive hemagglutination, and gel diffusion.

Morphologically intact and viable, free, P. berghei parasites were obtained by the sonic treatment of infected erythrocytes in an aqueous continuous flow system (Kreier et al., 1975). These parasites, when disrupted by freezing and thawing and brief sonic treatment yielded complement fixing antigens which were specific and potent (Prior and Kreier, 1972a). The antigens of the parasites were most stable if the whole parasites were stored frozen and the soluble antigen produced just before use.

Despite considerable effort we were unable to adapt our free parasite antigens to consistently satisfactory passive hemagglutination or gel diffusion procedures. Simple agglutination of the intact parasites also failed to occur consistently with serum from infected animals (Kreier, et al., 1965).

We compared the parasites obtained by the sonic system for freeing P. berghei to those obtained by distilled water lysis of infected erythrocytes and to those obtained by an ammonium chloride lysis technique (Martin, et al., 1971). The parasites obtained by the sonic technique were less damaged morphologically and yielded higher potency antigens than did those obtained by the other procedures (Prior, et al., 1973).

Plasmodium gallinaceum parasites freed in a nonaqueous medium from freeze-dried chicken erythrocytes were severely damaged. The yields of parasites were small and the antigens obtained from the parasites did not react with serum from infected chickens.

Attempts to adapt the aqueous continuous flow system to the Plasmodium gallinaceum-chicken erythrocyte system by use of buffers designed to protect nuclei were only slightly more successful than were attempts using the nonaqueous media system. Batch systems for freeing Plasmodia using sonic energy produced poor yields. The wave length of the sonic energy had little to do with the release (Prior and Kreier, 1977).

Work was carried out in our laboratory on elucidation of mechanisms of erythrocyte destruction in animals with malaria.

The work on erythrocyte destruction mechanisms indicated that it is very difficult to estimate accurately the actual numbers of parasites in the animal and the amount of erythrocyte destruction. This is partly because of retention of parasitized erythrocytes in internal capillary beds and partly because erythrocytes destroyed because of merozoite contact would not be counted as parasitized (Kreier and Leste, 1967, 1968; Wright and Kreier, 1969; Kreier, 1969; Kreier et al., 1972, 1972c; Swann and Kreier, 1972).

The environment in which the erythrocyte finds itself in the infected animal is probably a major factor responsible for premature erythrocyte destruction. This may be concluded from the fact that nonparasitized erythrocytes from infected animals transferred to noninfected animals may have normal life spans (Kreier and Leste, 1968). The major factors in the infected animal responsible for shortening erythrocyte life span are plasma ion imbalance and increased plasma acidity and viscosity, which result, in part, from increased plasma globulin concentration, particularly immunoglobulins (Kreier et al., 1966). These immunoglobulins include autoantibodies directed against damaged erythrocytes (Kreier et al., 1966; Gautum et al., 1970) and nucleic acids (Kreier and Dilley, 1969). The autoantibodies bind to damaged erythrocytes in vivo (Kreier and Ristic, 1964; Kreier et al., 1966; Kreier, 1969) and are specific for lipid components in the damaged erythrocyte's membranes (Seed and Kreier, 1969).

Changes in plasma phospholipid and cholesterol concentrations also occur. These, in turn, affect the membrane levels of these materials (Seed and Kreier, 1972).

Proteolytic enzymes of host and possibly parasite origin also appear to be present in the plasma and on erythrocytes; those affect the erythrocyte membranes (Kreier, et al., 1966), particularly the ATPase pump systems (Seed and Kreier, 1972). The changes in the erythrocyte membranes and the abnormality in the plasma of the infected animal are major factors in the destruction of nonparasitized erythrocytes during malaria. A final factor in the destruction of nonparasitized erythrocytes in malarious animals stems from the production of many short-lived stress reticulocytes by the animal attempting to compensate for the massive erythrocyte loss caused by infection (Kreier, et al., 1972b; 1972c).

We have adapted the previously developed continuous flow ultrasound procedure for freeing Plasmodium berghei to Babesia microti and B. rodhaini. We have studied the free parasites by complement fixation techniques, by morphologic means, and by biophysical means (Gravely and Kreier, 1976; Abdalla et al., 1978).

Babesia microti antigens fix complement with homologous antiserum to high titers and titers persist in recovered animals. Babesia microti antigens fix complement with serum for hamsters acutely infected

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with Plasmodium berghei but not with serum from recovered animals. The acute serum, moreover, fixed complement with erythrocyte membrane antigens but serum from recovered hamsters did not.

We have conducted a number of serologic tests by complement fixation which showed that Babesia microti, B. rodhaini and Plasmodium berghei antigens do not cross react (Zukerman, 1976). Autoantibodies to erythrocyte antigens were probably responsible for the cross reactions by the acute serum.

Immune serum passively protected hamsters from B. microti infection (Abdalla et al., 1978a).

The free parasites lent themselves to types of morphological studies not possible with intraerythrocytic parasites. From studies of free parasites combined with studies of intraerythrocytic parasites we have demonstrated that plasmodial and babesial merozoites are very similar in gross shape and membrane structure. It was demonstrated that both types of merozoites have a subplasmalemmal reticular network of a highly ordered nature. On the free Babesia preparations it was also possible to follow the sequence of events in bud formation. A change of the trophozoite membrane in the region of the bud so that it assumed the characteristics of the merozoite pellicle was demonstrated to be an early event in bud formation (Seed et al., 1971; 1973; Kreier et al., 1975).

In the course of our studies using free parasites the general topography and surface ultrastructure of intra- and extraerythrocytic malaria parasites of two species (Plasmodium gallinaceum and P. berghei) were examined by scanning and transmission electron microscopy utilizing metal-shadowed, carbon-replicated, and freeze-cleaved preparations. Standard thin sections were also examined in order to compare surface to cross-sectional images of the cytoplasmic bounding layers. Small extracellular parasites, which were either naturally free or liberated from infected red cells by ultrasonication, were consistently spherical to ovoid in shape, ranging in size from 1.0-1.5 μm . Larger parasites, 2-3 μm in diameter, were generally more pleomorphic. Free parasites were characterized by small surface protrusions or "buds" as well as a variety of invaginated areas. The larger invaginations, particularly those of the merozoites, were probably cytostomes.

Two distinct types of surface patterns were observed on intra- and extra-cellular parasites. A "table-lace" pattern was possessed by a large percentage of the small parasites which were probably merozoites. This type of patterned surface may have resulted from bulging of the surface where the thick inner membranous complex of the parasites' pellicle lay. Cross-sectional and topographical analysis of this complex suggests that it is a composite of flattened micro-tubules arranged in a series of distinct interlocking hexagonal rings.

The basic ring unit measured 0.25-0.30 μm in length to 0.14-0.17 μm in width, while each segment was approximately 0.12-0.14 μm wide. The variability of the measurements and the occasional observation of distorted basic pellicular units suggests that the component parts of this structure are highly elastic.

A second type of membrane pattern, common to both merozoites and trophozoites, was observed in freeze-cleaved preparations. This type of pattern, characterized by fine stippling, resulted from exposure of intramembranous particles, 25-35 nm in diameter, when membranes split along an interior cleavage plane. We believe that there are structural differences between parasite and host cell membranes which are characterized by differences in the size of these particles and in their distribution (Seed *et al.*, 1971; 1973; Seed and Kreier, 1976).

By particle electrophoretic means we have determined that merozoites have significant negative charges at physiological pH's and that these changes can be removed by extraction procedures which remove phospholipids, but not by neuraminidase treatment. Thus the charges on the parasites are born by phospholipids not sialic acid as are those on erythrocytes. We have also observed that trophozoites have a lower charge on their surfaces than merozoites; thus, these two stages of the parasite have membranes that differ not only morphologically but also physiologically (Seed and Kreier, 1976).

We have sensitized latex with soluble *P. berghei* antigen and shown that this antigen-sensitized latex can be specifically agglutinated by fresh serum from rats which have recovered from *P. berghei* infection. The antigen contained protein and carbohydrate. Delipidization of the antigen did not affect its specificity (Green, 1975).

We have described how the sonically freed parasites can be quantitated (Kreier *et al.*, 1975). Using these quantitative methods we have developed a test capable of detecting antimerozoite antibody in the serum of rats recovering from malaria (Hamburger and Kreier, 1975; 1976). We have demonstrated that immune serum contains opsonic antibody which markedly stimulates phagocytosis of merozoites by phagocytes from normal rats but does not contain antibody that stimulates phagocytosis of erythrocytes from infected rats (Chow and Kreier, 1972; Hamburger and Kreier, 1976; Green and Kreier, 1978). By fluorescent antibody procedures, we have shown that anti *Plasmodium berghei* antibody binds *in vitro* to free parasites but not to parasites in erythrocytes. We observed that antibody-coated parasites which are not capable of infecting rats do infect mice. Because of this observation and demonstration of phagocytosis *in vitro* of antibody-coated free parasites we proposed that antibody coating does not kill the parasites but prepares them for destruction by some other host defense mechanism, probably phagocytosis. We have shown that the antibody which attaches to free parasites *in vitro* contributes to the

host defense against infection and proved that antibody does not act against P. berghei when they are inside the rat erythrocyte (Hamburger and Kreier, 1975). From these experiments we made observations that suggested the possibility that the antibodies which bound to free parasites may elute fairly easily and thus may be of low avidity (Hamburger and Kreier, 1976a).

Our studies of the role of humoral factors in the rats' defense against P. berghei, particularly the implementation of the already induced immune response, indicate that antibody is important in antimalarial immunity. Experiments which concern the induction of the immune response rather than its implementation showed that in young rats progressive thymic involution was a feature of the disease but in the more resistant adult rats thymic hypertrophy occurred. The experiments also showed that in young and mature rats splenic T cell population changes followed thymic cell population changes. It was also revealed by these studies that in infected mature rats there was first a sharp decrease in complement receptor lymphocytes in the spleen and then an increase in their numbers, while in young rats there was only a slow and steady decrease in this cell type in the spleen. The order of the changes is evidence that effective immunity to malaria in rats is associated with B cell differentiation and that this differentiation is dependent on the presence of mature thymocytes (Gravely et al., 1976). We have shown clearly that adoptive transfer of immunity to rodent malaria is mediated by differentiated B cells and this supports the thesis that a major portion of the immunity to rodent malaria is effected by antibody, the product of the differentiated B cells (Gravely and Kreier, 1976).

Short term in vitro culture of Babesia microti was obtained. Immune hamster serum had an inhibitory effect on the growth of B. microti in vitro. It is suggested that the antibodies act by preventing penetration of erythrocytes by the parasite (Bautista and Kreier, 1979).

The effect on growth of B. microti of macrophages from normal or B. microti-recovered animals was tested in short-term cultures. Normal macrophages did not or only slightly inhibited parasite growth. When immune serum was added to the normal macrophage-containing cultures, inhibition greater than that produced by immune serum alone was observed. Macrophages from immune animals had an inhibitory effect on parasite growth even in the absence of immune serum. Inhibition of B. microti growth was greater in cultures containing immune serum and immune macrophages than in cultures containing either immune serum or immune macrophages alone. Cell free supernatants from B. microti cultures which contained immune macrophages or macrophages which had been stimulated by addition of immune serum inhibited parasite growth while cell-free supernatant from cultures which contained no macrophages or normal macrophages did not. At 72 and 96 hr. of culture erythrophagocytosis by macrophages was more prominent in immune serum containing B. microti cultures than in B. microti cultures not

containing immune serum. More macrophages were retained in the immune serum containing cultures than in cultures not containing immune serum. Alternate culture-hamster passage yielded a B. microti strain which grew better in hamster erythrocyte short-term cultures than did the original strain (Bautista and Kreier, 1980).

We have fractionated soluble P. berghei antigens by column and disc gel means and shown that the antigens of the small free parasite preparation differ from the antigens of the unselected intraerythrocytic parasite population at least quantitatively (Kreier et al., 1975).

We carried out a series of experiments on immunization of rats with a preparation of small free parasites. We delineated the conditions under which it is possible to immunize rats with small free parasites and to validly compare various treatments of the preparation such as adjuvant type and dose, immunogenicity of fractions of the preparation, and comparison of one preparation to other preparations (Saul and Kreier, 1977).

Soluble material was obtained from sonically freed plasmodia by three procedures. Two procedures, cryo-impacting and freeze-thawing, were evaluated for their ability to disrupt the parasites and release soluble material. The soluble materials obtained by these procedures were compared to materials washed from the surfaces of sonically freed parasites. Between 35 and 40% of the total parasite protein was solubilized by freeze-thawing or cryo-impacting. One cycle of freeze-thawing released nearly as much protein as could be released by this method, and additional cycles of freeze-thawing had little additional effect. Cryo-impacting solubilized only a small amount of protein in addition to that which was released by the cycle of freeze-thawing inherent in the procedure. Reductions in the packed cell volume of the material remaining after freeze-thawing or cryo-impacting indicate that the insoluble fragments are broken into smaller pieces as treatment is extended. Electron microscopy of 30 second cryo-impacted and three times freeze-thawed parasites revealed membrane fragments similar in appearance. Patterns obtained by polyacrylamide gel electrophoresis of the soluble material from freeze-thawed and cryo-impacted parasites were also similar, and approximately 13 protein bands were demonstrated. The material washed from the surfaces of the free parasites, on the other hand, resolved into only two to four major bands on the gel columns. In immunization studies, the soluble and insoluble fractions obtained by freeze-thawing or cryo-impacting and the material washed from the surfaces of the parasites all stimulated a protective immune response. On the basis of the amount of protein required to stimulate roughly comparable immunity, the soluble fraction obtained by freeze-thawing or cryo-impacting free parasites was about twice as potent an immunogen as was the insoluble fraction. The material obtained by gentle washing of the freed parasites was approximately 20 times as potent an immunogen as were the freed parasites and about 7 times as potent as the soluble material obtained by freeze-thawing or cryo-impacting (Grothaus and

Kreier, 1980). A similar purified fraction of plasmodia of human origin, if it can be obtained, may be a candidate for use as a vaccine against malaria.

We have identified protective antigens among the variety of antigens which the malaria parasite produces. This was done by immunization with free parasites and fractions of free parasites (Saul and Kreier, 1977; Grothaus and Kreier, 1980). The demonstration and characterization of macrophage-cytophilic and parasite-coating antibodies was a direct result of our efforts to study the protective factors in immune serum by absorption with parasites. We have determined that a macrophage-cytophilic antibody and an opsonic antibody in the immune serum mediates attachment of the parasites to the macrophages. The cytophilic antibody must attach to macrophages before it will react with the parasites. The macrophage-cytophilic antibody is an IgG₁ antibody while the antibody which binds to the parasites directly is IgG₂. The macrophage-cytophilic antibody may be eluted from macrophages by heating the sensitized macrophages at 56°C for 30 minutes or purified from hyperimmune serum by column chromatographic techniques. We have demonstrated that the macrophage-cytophilic antibody and the parasite coating antibody act synergistically in vivo to bring about destruction of the parasites, while alone the cytophilic antibody is almost ineffective in protecting the host (Green and Kreier, 1978).

I have prepared a critical review of the procedures for the isolation and characterization of antigens of plasmodia (Kreier, 1977). This review provides an analysis of the problems confronting those attempting to isolate antigenic materials from plasmodia. I have carried out research on how the plasmodium defends itself from the host's response. These studies show clearly the importance of the merozoite capsule in the resistance of the blood-inhabiting stages of plasmodia to the immune responses of the host (Brooks and Kreier, 1978). They also define the role of antibody and macrophages in the host's response to plasmodial infection (Chow and Kreier, 1972; Green and Kreier, 1978).

Our attempts to identify which antigens are protective by immunization have demonstrated that a soluble component of Plasmodium berghei is significant in inducing immunity, and we have tentatively identified this soluble component as surface coat material. Thus our work on identification of protection stimulating antigens in Plasmodium berghei has been successful. The knowledge obtained from these studies must now be applied to development of a vaccine against malaria of man.

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